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ORIGINAL ARTICLE

Determination of repaglinide in human plasma by high-performance liquid chromatography–tandem mass spectrometry[☆]

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Abstract A rapid and sensitive method based on high-performance liquid chromatography–tandem mass spectrometry (LC–MS/MS) has been developed for the determination of repaglinide in human plasma. The analyte and internal standard (I.S.), diazepam, were extracted from plasma (25 µL) by liquid–liquid extraction with diethyl ether–dichloromethane (60:40, v/v) and separated on a XDB-C₁₈ column using acetonitrile–ammonium acetate buffer (pH 6.8, 0.01 mol/L) as mobile phase. The retention times of repaglinide and I.S. were 1.95 and 2.35 min, respectively. Detection was carried out using API 4000 mass spectrometer with an ESI interface operating in the multiple reaction monitoring (MRM) mode. The assay was linear over the concentration range 0.050–50 ng/mL with a limit of detection (LOD) of 0.010 ng/mL. Intra- and inter-day precisions (as relative standard deviation, R.S.D.) were ≤5.07% and ≤11.2%, respectively, and accuracy

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(as relative error, R.E.) was from -0.593% to -1.26% . The assay was successfully applied to a pharmacokinetic study involving a single oral administration of a tablet containing 2 mg repaglinide to each of 10 healthy volunteers.

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1. Introduction

An increasing number of people worldwide suffer from type 2 diabetes mellitus (T2DM). Although this chronic disease can be controlled through diet, exercise and hypoglycaemic drugs, the control of blood glucose and reversal of insulin resistance is never adequate to avoid severe long-term complications such as cardiovascular disease, peripheral vascular disease, stroke, diabetic neuropathy, amputations, renal failure and blindness¹. Accordingly, research is focusing on new targets and therapeutic agents to reduce morbidity and mortality and

the need for insulin injections. In the development of such agents, sensitive analytical methods are required for their quantitation in clinical trials.

Repaglinide (2-ethoxy-4-[2-[[[(1*S*)-3-methyl-1-(2-piperidin-1-ylphenyl) butyl] amino]-2-oxo-ethyl] benzoic acid) (Fig. 1), a meglitinide analog, is an oral hypoglycemic agent used for the management of T2DM. It is particularly useful when metformin cannot be used due to adverse effects, when metformin fails to adequately control blood glucose levels or when there is a need for flexible dosing. It may also have an advantage in diabetic patients with renal impairment since it is eliminated

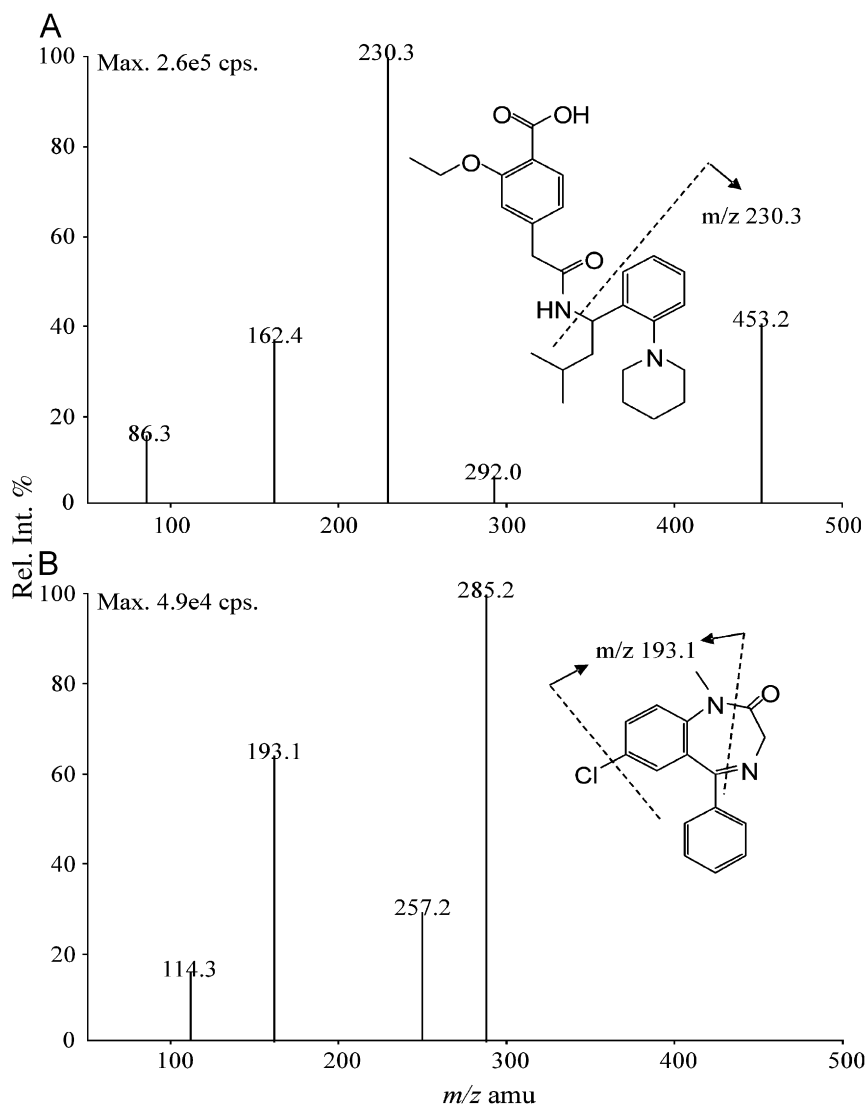


Figure 1 Full-scan product ion mass spectra of $[M+H]^+$ ions of (A) repaglinide and (B) diazepam.

through biliary excretion². It is characterized by relatively short duration of action and as monotherapy is usually administered at a dosage of 0.5–4 mg three times daily. The major metabolites of repaglinide are a dicarboxylic acid, an aromatic amine, or acyl glucuronide³. A previous study reported that the major metabolites of repaglinide have no hypoglycaemic activity⁴.

Several methods have been applied to determine the repaglinide in human biological samples involving competitive solid-phase enzyme immunoassay⁵, HPLC with electrochemical⁶, ultraviolet⁷ or diode-array detection⁸. The LC–MS/MS method has been developed to determine the repaglinide concentration in monkey plasma⁹ and equine plasma and urine¹⁰, but the LLOQ in the method described by Wang and Miksa⁹ is 1 ng/mL, which is not sufficient for clinical study. For the same reason, the method described by Ho et al.¹⁰ is not sensitive enough, either. In addition, the lack of matrix effect evaluation and stability evaluation that is very important in pharmacokinetic study¹¹, may preclude its further utilization in clinical trials. There is also an LC–MS/MS¹² method reported for the quantitation of repaglinide in human plasma, and the advantage of our method is using a relative small volume of plasma (25 μ L compared to 200 μ L) but more sensitive. In this paper we reported a sensitive LC–MS/MS method (LLOQ of 0.050 ng/mL) requiring a small volume of plasma (25 μ L), simple sample preparation and short running time (2.6 min). The method has been successfully applied to a pharmacokinetic study in healthy volunteers given a single oral dose of a repaglinide 2 mg tablet.

2. Materials and methods

2.1. Chemicals and reagents

Repaglinide (purity >99.0%) was supplied by Beijing Beilu Pharmaceutical Co., Ltd. (Beijing, China). Diazepam (purity >99.0%) for use as internal standard (I.S.) was purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). HPLC grade acetonitrile and methanol were purchased from Fisher Scientific (Fair Lawn, NJ, USA). All other reagents were of analytical grade and were used without further purification. Distilled water, prepared from demineralized water, was used throughout the study. Blank (drug free) human plasma was obtained from the Changchun Blood Donor Service (Changchun, China).

2.2. LC–MS/MS conditions

Liquid chromatography employed an Agilent 1100 series HPLC system (Agilent, Palo Alto, CA, USA), including a binary pump, an autosampler, and a column oven maintained at 35 °C. Chromatography utilized a Zorbax Eclipse XDB-C₁₈ column (150 mm \times 4.6 mm i.d., 5 μ m) with a mobile phase consisting of 70% acetonitrile and 30% ammonium acetate buffer (pH 6.8, 0.01 mol/L) at a flow rate of 1.2 mL/min. An approximately 1:1 split of the column elute was included allowing 0.6 mL/min to enter the mass spectrometer.

Mass spectrometric detection utilized an API 4000 mass spectrometer (Applied Biosystems Sciex, Ontario, Canada) equipped with an electrospray ionization (ESI) source operated

in the positive ion mode. Detection was carried out at unit resolution for both Q1 and Q3 with a dwell time of 200 ms per channel. Multiple reaction monitoring (MRM) transitions were m/z 453.2 \rightarrow 230.3 and m/z 453.2 \rightarrow 162.4 for repaglinide and m/z 285.2 \rightarrow 193.1 for diazepam. Optimized MS parameters were as follows: Curtain gas, gas 1 and gas 2 (all nitrogen) 15, 40 and 40 units, respectively; ion spray voltage 4000 V; source temperature 450 °C; declustering potentials (DP) 55 V for repaglinide and 80 V for I.S.; collision energies (CE) 37 eV (m/z 453.2 \rightarrow 230.3) and 30 eV (m/z 453.2 \rightarrow 162.4) for repaglinide and 42 eV for diazepam.

2.3. Calibration and quality control (QC) samples

Stock solutions of repaglinide (1.0 mg/mL) and I.S. (1.0 mg/mL) were prepared in methanol. A series of standard solutions of repaglinide were prepared by diluting the stock solution with methanol–water (50:50, v/v) to give concentrations of 0.050, 0.15, 0.50, 1.5, 5.0, 15 and 50 ng/mL. Calibration samples were prepared by spiking 25 μ L blank plasma samples with 25 μ L aliquots of standard solutions. Low, medium and high concentration QC samples (0.15, 1.5, 15 ng/mL) were prepared independently in the same way. An I.S. working solution with concentration 150 ng/mL was also prepared in methanol–water (50:50, v/v). Samples were stored at 4 °C when not in use.

2.4. Sample preparation

Frozen human plasma samples stored at –80 °C were thawed at room temperature and vortexed for 30 s. To 25 μ L human plasma in a glass tube, 25 μ L I.S. working solution, 25 μ L methanol–water (50:50, v/v) (or a calibration sample or QC sample) and 3 mL diethyl ether–dichloromethane (60:40, v/v) were added. The mixture was vortex-mixed for 30 s and centrifuged at 1500 $\times g$ for 5 min after which the supernatant was transferred to a clean glass tube and evaporated to dryness at 40 °C under a gentle stream of nitrogen. The residue was reconstituted in 100 μ L mobile phase of which 20 μ L was injected into the LC–MS/MS system.

2.5. Assay validation

Assay validation was performed according to FDA guidelines¹¹. Linearity was assessed by weighted ($1/x^2$) least-squares linear regression of analyte–I.S. peak area ratios based on three independent calibration curves prepared on three separate days. Precision (as relative standard deviation, R.S.D.) and accuracy (as relative error, R.E.) were evaluated by assay of six replicate QC samples on three different days. The lower limit of quantitation (LLOQ) was defined as the lowest concentration of analyte that could be determined with precision of $\leq 20\%$ and accuracy within $\pm 20\%$. The limit of detection (LOD) was taken as the concentration with signal-to-noise ratio of 3. Selectivity of the method was carried out by comparing the chromatograms of six different batches of blank human plasma with corresponding spiked plasma samples. Recoveries of analyte and I.S. were determined by comparing the peak areas in extracted QC samples with those in post-extraction blank samples spiked at the corresponding concentrations. Matrix effects for repaglinide were evaluated by comparing peak areas in extracted samples of blank plasma

spiked with low, medium and high concentrations with those from pure standard solutions at the same concentrations. Matrix effects for the I.S. were assessed at the concentration used in the assay in the same way. A stability study involved assay of QC samples after storage at -80°C for one month, at room temperature for 6 h and after three freeze-thaw cycles. Post-processing stability of reconstituted solutions in the autosampler at room temperature for 8 h was also assessed.

2.6. Pharmacokinetic study

The method was applied to a pharmacokinetic study involving 10 healthy volunteers given a single oral dose of a repaglinide 2 mg tablet. The clinical protocol was approved by the Ethics Committee of Peking University First Hospital, China. All volunteers gave written informed consent before entering the study. During the study, no subject was allowed to take alcohol or any other medication. Blood samples were collected into heparinized glass tubes before and at 0.25, 0.5, 0.75, 1,

1.5, 2, 2.5, 3, 4, 5 and 6 h after dosing. Plasma was separated immediately by centrifugation at $3000\times g$ for 10 min and stored at -80°C until analysis. Pharmacokinetic parameters were calculated using Topfit 2.0.

3. Results and discussion

3.1. Method development

In optimizing MS conditions, it was found that repaglinide ionized in both positive and negative modes but gave much higher ionization efficiency in the positive ion mode. Full-scan product ion mass spectra of repaglinide and I.S. are shown in Fig. 1. The MRM transition of repaglinide at m/z $453.2 \rightarrow 230.3$ was more intense than that at m/z $453.2 \rightarrow 162.4$ leading to the former being used as quantifier and the latter as qualifier.

In developing the chromatographic conditions, a number of reversed phase HPLC columns (Hypersil ODS2, Zorbax extend

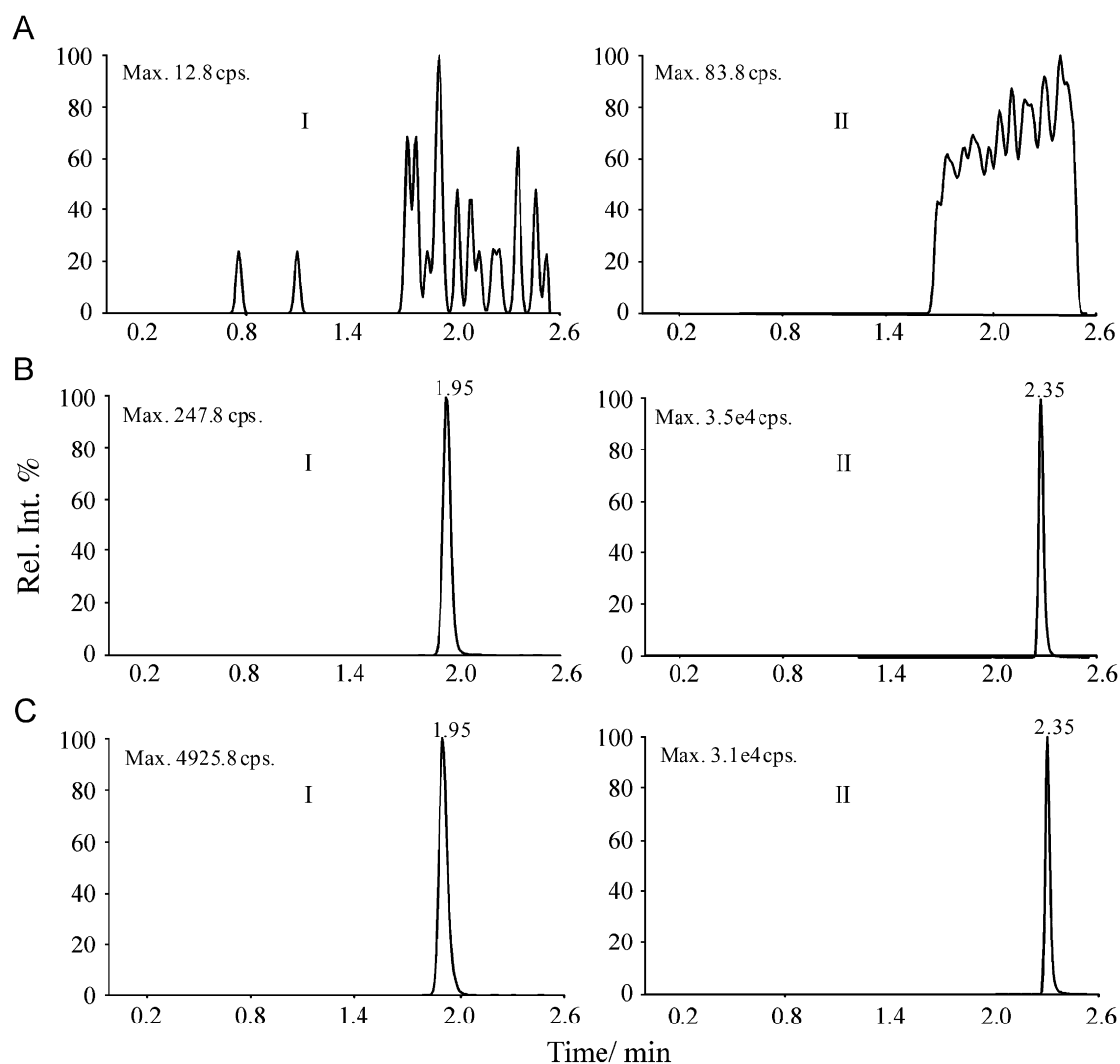


Figure 2 Typical MRM chromatograms of (I) repaglinide and (II) diazepam in human plasma: (A) blank plasma; (B) blank plasma spiked with 0.050 ng/mL repaglinide and 150 ng/mL I.S. and (C) a plasma sample from a human volunteer 4 h after oral administration of a repaglinide 2 mg tablet.

C₁₈ and Zorbax Eclipse XDB-C₁₈) were evaluated. Mobile phase combinations of acetonitrile, methanol, water and ammonium acetate with or without formic acid were also evaluated. The Zorbax Eclipse XDB-C₁₈ gave the best combination of sensitivity and peak shapes; acetonitrile produced a shorter retention time than methanol; ammonium acetate produced better peak shapes than water; addition of formic acid decreased sensitivity and extended the retention times. On this basis a mixture of 70% acetonitrile and 30% ammonium acetate buffer (pH 6.8, 0.01 mol/L) was adopted as the mobile phase.

In choosing the I.S., a number of compounds (diazepam, tramadol, metformin, acetaminophen, diphenhydramine, huperzine A, codeine and telmisartan) were evaluated. Diazepam was chosen because, although both it and diphenhydramine showed similar chromatographic behavior to repaglinide, diphenhydramine was susceptible to tailing. Using a flow rate of 1.2 mL/min, the retention times of repaglinide and I.S. were 1.95 and 2.35 min, respectively.

3.2. Sample preparation

Liquid–liquid extraction was chosen for sample preparation based on its reliability and cost effectiveness relative to solid phase extraction. A number of organic solvents (diethyl ether, dichloromethane, hexane, isopropanol and ethyl acetate) alone and in different combinations were evaluated. Diethyl ether–dichloromethane (60:40, v/v) was found to give high recovery of both analyte and I.S. with minimal matrix effects.

3.3. Assay validation

Fig. 2 showed representative chromatograms of blank plasma, plasma spiked with repaglinide at the LLOQ (0.050 ng/mL) and a plasma sample from a healthy volunteer after administration of repaglinide. The assay was free of interference from endogenous substances in plasma.

Calibration curves showed good linearity in the concentration range 0.050–50 ng/mL with an LLOQ of 0.050 ng/mL and LOD of 0.010 ng/mL. The accuracy and precisions of LLOQ were listed in Table 1. A typical regression equation for the calibration curve was $y = 4.89 \times 10^{-2}x + 2.12 \times 10^{-4}$, $r = 0.9982$. Intra- and inter-day precisions were below 5.07% and 11.2%, respectively. The accuracy ranged from −0.593% to −1.26% (Table 1).

The recoveries of repaglinide at 0.15, 1.5 and 15 ng/mL were $98.2\% \pm 3.4\%$, $99.1\% \pm 1.4\%$ and $94.0\% \pm 5.2\%$, respectively. The recovery of the I.S. was $95.1\% \pm 5.1\%$. Matrix

effects of repaglinide at low, medium and high QC concentrations were $101.6\% \pm 1.3\%$, $92.7\% \pm 6.1\%$ and $90.2\% \pm 1.7\%$, respectively. A matrix effects study of the I.S. gave a result of $95.5\% \pm 3.7\%$. Table 2 presented the stability data. Repaglinide proved to be stable under all the conditions tested.

Table 2 Stability data for repaglinide under various conditions ($n=3$).

Storage conditions	Concentration (ng/mL)	
	Nominal	Found (mean \pm SD)
Room temperature for 6 h (in human plasma)	0.150	0.153 ± 0.00755
	1.50	1.47 ± 0.0666
	15.0	15.1 ± 0.569
Freezing for 30 days at -80°C (in human plasma)	0.150	0.152 ± 0.00721
	1.50	1.54 ± 0.0757
	15.0	13.9 ± 0.208
Three freeze/thaw cycles (in human plasma)	0.150	0.145 ± 0.0144
	1.50	1.58 ± 0.0100
	15.0	14.5 ± 0.289
Post-extraction stability at room temperature for 8 h	0.150	0.141 ± 0.00586
	1.50	1.58 ± 0.0351
	15.0	15.6 ± 0.458

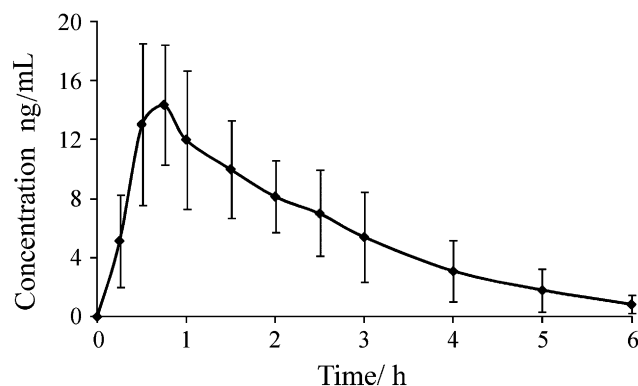


Figure 3 Mean plasma concentration–time curve for repaglinide after oral administration of a repaglinide 2 mg tablet (data are mean \pm SD, $n=10$).

Table 1 Accuracy and precision for the analysis (data are based on assay of 6 replicates on 3 different days) and LLOQ of repaglinide in human plasma.

Concentration (ng/mL)		Precision (RSD/%)		Accuracy (RE/%)
Nominal	Found (mean \pm SD)	Intra-day	Inter-day	
0.150	0.148 ± 0.00836	5.07	8.78	−1.15
1.50	1.48 ± 0.0795	3.98	11.2	−1.26
15.0	14.9 ± 0.800	4.72	8.80	−0.593
0.0500	0.0512 ± 0.00263		5.15	2.15

Table 3 Pharmacokinetic parameters for ten healthy volunteers after oral administration of a repaglinide 2 mg tablet.

Volunteer number	C_{\max} (ng/mL)	t_{\max} (h)	$t_{1/2}$ (h)	AUC _{0–6 h} (ng · h/mL)	CL (mL/min)	MRT (h)
1	8.87	0.50	0.90	21.77	1500	1.97
2	18.10	0.50	1.22	55.47	562	2.54
3	16.10	1.50	1.16	37.96	842	2.05
4	11.00	0.50	1.17	28.02	1150	1.94
5	23.70	0.50	1.08	44.31	735	1.59
6	17.40	1.00	1.20	37.87	843	2.08
7	15.30	0.75	1.22	40.47	776	2.17
8	16.20	0.50	0.87	33.38	983	1.87
9	13.90	0.75	0.95	23.64	1390	1.51
10	12.20	0.75	1.12	24.77	1300	1.92
Mean	15.28	0.73	1.09	34.77	1008	1.96
±SD	4.16	0.32	0.13	10.60	312	0.29

3.4. Pharmacokinetic study

The mean plasma concentration–time profile was shown in Fig. 3 and corresponding pharmacokinetic parameters were listed in Table 3. The time to reach maximum plasma concentration (t_{\max}) and the elimination half-life ($t_{1/2}$) were similar to the values previously reported for repaglinide given as a single oral 2 mg dose⁵. The maximum plasma concentrations (C_{\max}) and areas under the plasma concentration–time curve (AUC_{0–t}) were in the range 8.87–23.7 ng/mL and 21.8–55.5 ng h/mL, respectively, indicating high inter-individual variability. This phenomenon has been attributed to the fact that repaglinide is a substrate of the organic anion transporting polypeptide (OATP) and of CYP2C8 both of which exhibit genetic polymorphism^{13,14}. The results confirm that the assay is suitable for clinical pharmacokinetic studies of repaglinide.

4. Conclusions

A rapid and sensitive LC–MS/MS method for the determination of repaglinide in human plasma has been developed and validated. The method has high sensitivity (LLOQ of 0.050 ng/mL) and requires only a small volume of human plasma (25 µL). The method also exhibited excellent performance in terms of recovery and matrix effect. Simple sample preparation and short run time allow high sample throughput for clinical studies.

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